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<b>(21) International Application Number:</b> PCT/FI95/00480 <b>(22) International Filing Date:</b> 7 September 1995 (07.09.95) <b>(30) Priority Data:</b> 954195 7 September 1995 (07.09.95) FI <b>(71) Applicant (for all designated States except US):</b> CULTOR OY [FI/FI]; Kyllikinportti 2, FIN-00240 Helsinki (FI). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> VIRTANEN, Erkki [FI/FI]; Niemenmäentie 3-5 K 79, FIN-00350 Helsinki (FI). PEHU, Eija [FI/FI]; Mechelininkatu 25 A 7, FIN-00100 Helsinki (FI). <b>(74) Agent:</b> OY KOLSTER AB; Iso Roobertinkatu 23, P.O. Box 148, FIN-00121 Helsinki (FI).		<b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, MW, SD, SZ, UG), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>In English translation (filed in Finnish).</i>
<b>(54) Title:</b> IMPROVING THE YIELD OF PLANTS  <b>(57) Abstract</b>  The invention relates to the exogenous use of betaine to improve the yield of grain legumes. According to the invention, betaine is used to improve the yield especially under stress conditions. The invention also relates to grain legumes treated exogenously with betaine, and particularly to the seeds of such plants.		

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## Improving the yield of plants

### Technical field

5           The invention relates to the use of betaine to  
improve the yield of plants. The invention relates  
especially to the use of betaine to improve the yield  
of grain legumes. According to the invention, the yield  
10           can be improved both under normal and stress conditions,  
i.e. when the conditions are poor due to e.g. drought,  
high salinity, low temperatures, humidity or  
environmental pollutants interfering with the growth.  
The invention also relates to grain legumes treated with  
betaine and to the parts thereof, especially seeds, and  
15           to products prepared from these.

### Background

          The environment and conditions for growth  
considerably affect the yield of plants. Optimum growth  
environment and conditions usually result in a yield  
20           that is large in quantity and high in quality. Under  
poor growth conditions both the quality and the quantity  
naturally deteriorate.

          The physiological properties of a plant are  
preferably manipulated by means of breeding, both with  
25           traditional breeding methods and for example with  
genetic manipulation.

          Several different solutions concerning  
cultivation technique have been developed to improve the  
growth conditions and yield of plants. Selecting the  
30           right plant for the right growth location is self-  
evident for a person skilled in the art. During the  
growing season plants may be protected with mechanical  
means by utilizing for example different gauzes or  
plastics or by cultivating the plants in greenhouses.  
35           Irrigation and sprinkler irrigation, fertilizers and

plant nutrients are generally used in order to improve the growth. Surfactants are often used in connection with applying pesticides, protective agents and minerals. Surfactants improve the penetration of these substances into plant cells, thereby enhancing and increasing the effect of the aforementioned agents and simultaneously reducing their harmful effects on the environment. However, different methods of cultivation technique are often laborious and impractical, their effect is limited (the economical size of a greenhouse, the limited protection provided by gauzes, etc.), and they are also far too expensive on a global scale. No economically acceptable chemical solutions for protecting plants from stress conditions have been described so far.

Water supply is more important than any other environmental factor for the productivity of a crop, even though the sensitivity of plants to drought varies. Irrigation is usually utilized to ensure sufficient water supply. However, there are significant health and environmental problems related to irrigation, for example a sharp decrease in water resources, deterioration of water quality and deterioration of agricultural lands. It has been calculated in the field that about half of the artificially irrigated lands of the world are damaged by waterlogging and salinization. An indication of the significance and scope of the problem is that there are 255 million hectares of irrigated land in the world, and they account for 70% of the total world water consumption. In the United States alone, there are over 20 million hectares of irrigated land mainly in the area of the 18 western states and in the southeastern part of the country. They use 83% of the total water consumption for irrigation alone. It can also be noted that the use of irrigation

water increases every year especially in industrial countries. In addition to these problems, another drawback of irrigation is the high cost.

5 Another serious stress factor is the salinity of soil. The salinity of soil can be defined in different ways; according to the general definition, soil is saline if it contains soluble salts in an amount sufficient to interfere with the growth and yield of several cultivated plant species. The most common of the  
10 salts is sodium chloride, but other salts also occur in varying combinations depending on the origin of the saline water and on the solubility of the salts.

It is difficult for plants growing in saline soil to obtain a sufficient amount of water from the  
15 soil having a negative osmotic potential. High concentrations of sodium and chloride ions are toxic to plants. An additional problem is the lack of minerals, which occurs when sodium ions compete with potassium ions required, however, for cell growth, osmoregulation and pH stabilization. This problem occurs especially  
20 when the calcium ion concentration is low.

The productivity of plants and their sensitivity to the salinity of soil also depend on the plant species. Halophytes require relatively high sodium  
25 chloride contents to ensure optimum growth, whereas glycophytes have low salt tolerance or their growth is considerably inhibited already at low salt concentrations. There are great differences even between different cultivars of a cultivated plant species. The  
30 salt tolerance of one and the same species or cultivar may also vary depending for example on the stage of growth. In the case of low or moderate salinity, the slower growth of glycophytes cannot be detected in the form of specific symptoms, such as chlorosis, but it is  
35 shown in the stunted growth of the plants and in the

colour of their leaves that is darker than normal. Moreover, the total leaf area is reduced, carbon dioxide assimilation decreases and protein synthesis is inhibited.

5                   Plants can adapt to some extent to growth and stress conditions. This ability varies considerably depending on the plant species. As a result of the  
10                   aforementioned stress conditions, certain plants begin to produce a growth hormone called abscisic acid (ABA), which helps the plants to close their stomata, thus  
15                   reducing the severity of stress. However, ABA also has harmful side effects on the productivity of plants. ABA causes for example leaf, flower and young fruit drop and inhibits the formation of new leaves, which naturally  
15                   leads to reduction in yield.

                  Stress conditions and especially lack of water have also been found to lead to a sharp decrease in the activity of certain enzymes, such as nitrate reductase and phenylalanine ammonium lyase. On the other hand, the  
20                   activity of alpha-amylase and ribonuclease increases. No chemical solutions, based on these findings, to protect plants have been described so far.

                  It has also been found that under stress conditions certain nitrogen compounds and amino acids, such as proline and betaine, are accumulated in the  
25                   regions of growth of certain plants. The literature of the art discusses the function and meaning of these accumulated products. On the one hand it has been proposed that the products are by-products of stress and  
30                   thus harmful to the cells, on the other hand it has been estimated that they may protect the cells (Wyn Jones, R.G. and Storey, R.: *The Physiology and Biochemistry of Drought Resistance in Plants*, Paleg, L.G. and Aspinall, D. (Eds.), Academic Press, Sydney, Australia, 1981).

Zhao et al. (in *J. Plant Physiol.* 140 (1992) 541 - 543) describe the effect of betaine on the cell membranes of alfalfa. Alfalfa seedlings were sprayed with 0.2M glycinebetaine, whereafter the seedlings were uprooted from the substrate, washed free of soil and exposed to temperatures from -10°C to -2°C for one hour. The seedlings were then thawed and planted in moist sand for one week at which time regrowth was apparent on those plants that had survived. Glycinebetaine clearly improved the cold stability of alfalfa. The effect was particularly apparent at -6°C for the cold treatment. All controls held at -6°C for one hour died, whereas 67% of the seedlings treated with glycinebetaine survived.

Itai and Paleg (in *Plant Science Letters* 25 (1982) 329 - 335) describe the effect of proline and betaine on the recovery of water-stressed barley and cucumber. The plants were grown in washed sand, and polyethylene glycol (PEG, 4000 mol. wt.) was added to the nutrient solution for four days in order to produce water stress, whereafter the plants were allowed to recover for four days before harvesting. Proline and/or betaine (25 mM, p 6.2) was sprayed on the leaves of the plant either on the first or third day of the stress or immediately before harvesting. As regards barley, it was noted that betaine supplied either before or after the stress had no effect, whereas betaine added in the end of the stress was effective. Proline had no effect. No positive effect was apparent for cucumber. On the contrary, it was found out that both betaine and proline had a negative effect.

Experiments aiming at clarifying the effects of betaine and proline on plants have thus yielded contradictory results. There are no commercial applications based on these results.

**Brief description of the invention**

The purpose of the present invention was to find a way to partially replace irrigation so that the amount and quality of the yield could be simultaneously ensured. Another purpose of the invention was to find a way to protect plants also under other stress conditions, such as during high salinity often connected with drought, at low temperatures, etc. Moreover, a further aim was to find a way to increase the yield under normal conditions without utilizing methods that would consume environmental resources or harm the environment.

In connection with the present invention it has been proved that the yield of grain legumes can be considerably improved by means of exogenously applied betaine. Betaine has been found to be effective in improving the yield both under normal and stress conditions, and it has no such detrimental effects as the side effects of ABA. Betaine application makes it possible to considerably reduce for example the need for artificial irrigation, thus saving the environment and cutting down the costs to a great extent. An advantageous feature of the invention is also the decrease of the antinutrient concentration of plants as a result of the betaine application. A good example of this is the low alkaloid content of lupins treated with betaine, i.e. about half of the normal level.

The invention thus relates to the exogenous use of betaine to improve the yield of grain legumes. The invention relates especially to the use of betaine to improve the seed yield of grain legumes.

According to the invention, betaine is used exogenously to improve the yield of grain legumes both under normal and stress conditions.



The invention also relates to the exogenous use of betaine to reduce the antinutrient content of grain legumes, especially to reduce the alkaloid content of lupin.

5           The invention further relates to grain legumes treated exogenously with betaine and to the parts thereof, particularly seeds, and to their use as such and for example in food, animal feed and forage industries.

10           The invention also relates to a method of improving the yield of grain legumes, in which method betaine is exogenously applied to growing grain legumes.

15           The invention further relates to a method of reducing the antinutrient content of grain legumes, in which method betaine is exogenously applied to growing grain legumes. The invention especially relates to a method of reducing the alkaloid content of lupins, in which method betaine is exogenously applied to growing lupins.

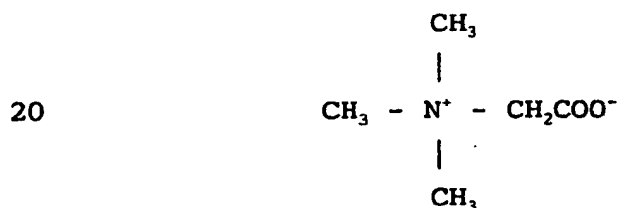
20           Betaine is applied to the plant in either one or several dosages. The application may be performed for example by spraying together with some other spraying of for example a pesticide, if desired. Betaine used according to the invention is transported to plant  
25           cells, where it actively regulates the osmotic balance of the cells and also participates in other processes of cell metabolism. A plant cell treated with betaine is more viable even when subjected to exogenous stress factors.

30           The betaine treatment according to the invention is economically advantageous, and the yield increases in an amount that is economically profitable and significant. The treatment does not produce significantly more work since it may be performed  
35           together with other sprayings, and it does not require

new investments in machinery, equipment or space. It should also be noted that betaine is a non-toxic natural product, which has no detrimental effects on the quality of the yield. Betaine is also a stable substance that remains in the plant cells and thereby has a long-standing effect.

#### Detailed description of the invention

Betaine refers to fully N-methylated amino acids. Betaines are natural products that have an important function in the metabolism of both plants and animals. One of the most common betaines is a glycine derivative wherein three methyl groups are attached to the nitrogen atom of the glycine molecule. This betaine compound is usually called betaine, glycinebetaine or trimethylglycine, and its structural formula is presented below:



Other betaines are for example alaninebetaine and prolinebetaine, which has been reported to for example prevent perosis in chicks. R.G. Wyn Jones and R. Storey describe betaines in detail in *The Physiology and Biochemistry of Drought Resistance in Plants* (Paleg, L.G. and Aspinall, D. (Eds.), Academic Press, Sydney, Australia, 1981). The publication is included herein by reference.

Betaine has a bipolar structure and it contains several chemically reactive methyl groups which it can donate in enzyme-catalyzed reactions. Most organisms can synthesize small amounts of betaine for example for the

methvl function, but they cannot react to stress by substantially increasing the production and storage of betaine. Best known organisms accumulating betaine are plants belonging to the *Chenopodiaceae* family, for example sugar beet, and some microbes and marine invertebrates. The main reason for the betaine accumulation in these organisms is probably that betaine acts as an osmolyte and thus protects the cells from the effects of osmotic stress. One of the main functions of betaine in these plants and microbes is to increase the osmotic strength of the cells when the conditions require this, for example in case of high salinity or drought, thus preventing water loss. Unlike many salts, betaine is highly compatible with enzymes, and the betaine content in cells and cell organelles may therefore be high without having any detrimental effect on the metabolism. Betaine has also been found to have a stabilizing effect on the operation of macromolecules; it improves the heat resistance and ionic tolerance of enzymes and cell membranes.

Betaine can be recovered for example from sugar beet with chromatographic methods. Betaine is commercially available from Cultor Oy, Finnsugar Bioproducts as a product that is crystalline water-free betaine. Other betaine products, such as betaine monohydrate, betaine hydrochloride and raw betaine-containing liquids, are also commercially available and they can be used for the purposes of the present invention.

According to the present invention, betaine is thus used exogenously to improve the yield of grain legumes, such as soybean, faba bean, green bean and other beans, pea, lupin, etc. According to the invention, betaine is used to improve the yield of grain legumes both under normal and stress conditions, i.e.

when the plants are subjected to periodic or continuous exogenous stress. Such exogenous stress factors include for example drought, high temperatures, high soil salinity, air pollution, such as ozone, nitrogen oxides, sulphur dioxide and sulphuric acid (acid rain), environmental poisons, herbicides, pesticides, etc. Treating plants subjected to stress conditions exogenously with betaine for example improves the adaptation of the plants to the conditions and maintains their growth potential longer, thereby improving the yield-producing capacity of the plants. Betaine is also a stable substance that remains in the plant cells. The positive effect of betaine is thereby long-standing and diminishes only gradually due to dilution caused by the growth.

Even though this reference and the claims use the word 'betaine', it is clear that according to the invention several different betaines can be used, if desired. It should also be noted that betaine is used here as a general term which thus covers different known betaines.

Betaine is applied to the plants in either one or several dosages. Application in a single dose is considered preferable. The amount used varies depending on the grain legume species and cultivar, and on the stage and conditions of growth. A useful amount may be for example about 0.1 to 20 kg of betaine per hectare. A preferable amount is thus for example about 1 to 6 kg of betaine per hectare. The amounts given here are only suggestive; the scope of the present invention thus contains all amounts that work in the manner described herein.

Any method suitable for the purpose may be used for the application of betaine. Betaine can be applied separately or together with other plant protectants,

pesticides or nutrients, such as fungicides and urea or micronutrients. Betaine can be applied easily for example by spraying. Foliar application of betaine and possible other agents through spraying is a preferable method which enables a more rapid response than methods involving root application. However, there may be different problems related to this method, such as low penetration concentrations in leaves with thick cuticles, run-off from hydrophobic surfaces, washing off by rain, rapid drying of the solution and leaf damage, and therefore other methods may also be used to apply betaine, if desired.

According to the invention, an aqueous solution of betaine is preferably used.

The time of the treatment according to the invention may also vary. If betaine is applied in a single dosage, the treatment is usually performed at an early stage of growth, for example on plants of about 5 to 20 cm, or when the leaves have just come out. If betaine is applied in several dosages, a new spraying is performed preferably in the beginning of flowering or when stress can be forecasted on the basis of the weather.

The betaine treatment according to the invention considerably improves the yield of grain legumes, for example the amount and quality of the yield. The treatment according to the invention can also reduce the need for artificial irrigation. The treatment according to the invention is economically advantageous and the increase in yield is economically profitable and significant. In connection with the invention it has been shown that for example lupin yield can be increased by over 28% with a suitable betaine dosage, for example about 6 kg/ha. It should also be noted that even though the amount of yield increases to a considerable extent,

the quality does not deteriorate. On the contrary, in connection with the present invention it has been shown that the antinutrient content of plants, for example the alkaloid content of lupins, considerably decreases as a result of the betaine application according to the invention.

High concentrations of alkaloids are poisonous to animal cells, and therefore a low alkaloid content in lupins is an important criterion of quality in view of lupin applications. One of the quality requirements for use in foodstuffs is that lupin seeds contain less than 0.02% of alkaloids. There has generally been a tendency to maintain the level of alkaloids as low as possible by selecting cultivars with a low alkaloid content. Since lupin cultivars with higher alkaloid contents produce higher yields, this approach has not been considered very advantageous. Eliminating alkaloids or reducing their amount during the processing of lupins has also been utilized, but this naturally increases the number of processing steps and the costs.

Most of the lupin yield is used as forage or in some other form as food for animals whereupon the advantages of lupin are its high contents of protein, amino acids and energy. The highest allowed alkaloid content for these applications is 0.04%. However, even with low concentrations alkaloids cause a bitter off-taste, wherefore animals tend to avoid eating forage or other feed containing alkaloids. The use of lupins in animal food and forage applications is therefore restricted due to their alkaloid content. It is also known that the alkaloid content of lupins increases under stress conditions.

The considerable decrease in the alkaloid content of lupins achieved with the betaine treatment according to the present invention is therefore a

significant additional advantage which is even more marked in view of the notable positive effect of betaine on the stress resistance of plants.

5 According to the invention, the yield of grain legumes can thus be improved both under normal and stress conditions, which in addition to drought include for example high salinity often connected with drought, high temperature, etc. Furthermore, the invention also makes it possible to grow grain legumes on lands that  
10 were previously considered unfit for cultivation.

The invention will be described in greater detail by means of the following examples. The examples are only provided to illustrate the invention, and they should not be considered to limit the scope of the  
15 invention in any way.

#### Example 1

##### Effect of betaine application on lupin yield

The effect of betaine application on lupin yield was examined at Murdoch University, Perth, Australia. The experiment was conducted under field  
20 conditions during the winter of 1994, which was cooler and rainier than usual, but during which water stress did occur, however.

The experiment was conducted according to a split-plot design utilizing plots of 8 m<sup>2</sup>. The plots were divided into four sub-plots that were treated with different betaine concentrations. The betaine concentrations used were 0 (control), 2 kg/ha, 4 kg/ha and 6 kg/ha. The soil was sandy (98% sand, 1% silt and  
25 1% clay) with a low nitrogen, phosphorus and potassium content and poor water and nutrient retention properties. The amount of irrigation was normal. The cultivar was Gungurru. The results are shown in Table  
30 1.

**Table 1**

Effect of betaine application on lupin yield

betaine concentration (kg/ha)	yield of lupin seeds	
	kg	% of control
0 (control)	0.979	100
2	0.992	101
4	1.048	107
6	1.253	128

The results show that the yield increased over the control in all the experiments conducted. The best results were obtained with a betaine application rate of 4 or 6 kg/ha.

**Example 2****Effect of betaine application on lupin yield under dry conditions**

The effect of betaine application on lupins growing under water stress was examined by repeating the experiment described in Example 1, but with a 50% reduction in irrigation from the optimum amount. The results are shown in Table 2.

**Table 2**

Effect of betaine application on lupin yield under water stress

betaine concentration (kg/ha)	yield of lupin seeds	
	kg	% of control
0 (control)	0.856	100
2	0.938	110
4	0.912	107
6	1.031	120



The yield also increased clearly in this experiment compared with the control. It can also be noted that utilizing the higher glycine betaine concentration of 6 kg/ha provided similar results with a low irrigation level (50%) as utilizing the lower betaine rate of 2 to 4 kg/ha with optimum irrigation (Example 1). This means that the same yield can be achieved by decreasing irrigation if a higher betaine application rate is used simultaneously.

#### Example 3

#### Effect of betaine application on the alkaloid content of lupins

The alkaloid content of lupins grown and treated according to Examples 1 and 2 was determined with the method described by Priddis [Journal of Chromatography, 261 (1983) 95 - 101]. The results are shown in Table 3.

Table 3

Effect of betaine application on the alkaloid content of lupin seeds

treatment	alkaloid content (%)
control I irrigation rate 50%	0.04
6 kg/ha betaine irrigation rate 50%	0.02
control II irrigation rate 100%	0.02
6 kg/ha betaine irrigation rate 100%	0.01

The results show a clear decrease in the total alkaloid content of lupins, which is a very surprising and positive result from the betaine application according to the invention.

**Example 4****Effect of betaine application on the early development of green bean seeds**

5 The effect of betaine on the germination frequency and rate of green bean seeds was examined using water as control. The green bean was of the type Spartan Arrow Bush Bean Lot #1987-3, produced by Northrup King Co. Three different test solutions were prepared for the experiments as follows:

10

Test solution	pH
A deionized water	7.01
B betaine (0.02 g/l)	6.34
C betaine (2 g/l)	6.80

15

Twenty green bean seeds were soaked for 24 hours in 330 ml of one of the aforementioned test solutions. The seeds were then dried on stainless steel screens and sown into soil with two seeds placed in each container. 20 The containers were then placed on a window ledge with a southern exposure to the sun, and they were watered daily with deionized water.

The early development of the seeds was followed by determining both the rate and frequency of 25 germination. The first measurements were performed ten days after the experiment began, and the second set of measurements was conducted 19 days after the beginning of the experiment. The results are shown in Table 4.

Table 4

Effect of betaine application on the early development of green bean seeds

Betaine concentration (g/l)	Germination frequency (%)		Average shoot length	
	Day 10	Day 19	(inch)	(% of control)
0 (control)	0	40	2.25	100
0.02	80	100	4.82	214
2	20	100	3.88	172

The results show that betaine promotes faster germination in green beans. Betaine also produced changes in the look of plants, for example the colour of leaves became dark green. The best results were achieved with the lower betaine rate of 0.02 g/l.

#### Example 5

#### Effect of betaine application on pea yield under stress conditions

The effect of betaine application on the yield of peas growing under stress conditions was examined in the following way. Peas were sown in 5 l plastic pots containing a mixture of peat and vermiculite in a ratio of 1:1. The plants were grown in greenhouses at a mean day/night temperature of 28°C/12°C and a relative humidity of between 42 and 45%. Supplemental illumination was provided for 17 hours a day with tungsten lamps (PAR 434  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ). Twenty seeds were sown per pot and they were later thinned to 10 plants per pot. The total number of pots used was twenty-four, twelve of which were exposed to drought, i.e. water stress, and twelve to high salinity, i.e. salt stress.

A completely randomised design was utilized in the experiment, with 4 replications.

5        Water stress (pF3) was imposed on half of the plants four weeks after seedling emergence. The pots were then grouped into 3 sets, each of which consisted of 4 pots, and each set was sprayed with either 25 ml of distilled water, 0.1M betaine solution or 0.3M betaine solution two weeks after the stress imposition.

10       To induce salt stress, 200 ml of 100 mM NaCl solution was applied to half of the pots every four days for five weeks after seedling emergence. The pots were grouped in 3 sets of 4 pots in each set, and they were sprayed with 25 ml of distilled water, 0.1M betaine solution or 0.3M betaine solution after the first  
15       administration of the NaCl solution. The NaCl treatment was repeated six more times after the betaine application.

20       At harvest, the total number of nodules, number of active nodules, number of pods, and leaf dry matter content were determined. The results are shown in Tables 5 and 6.



The smaller betaine rate of 0.1M thus had a positive effect both on the number of active nodules, number of pods and the leaf dry matter content when the pea was growing under dry conditions. The positive effect on pea growing under salt stress was even clearer. The higher betaine content of 0.3M had a positive effect on the number of nodules and leaf dry matter content of peas growing under salt stress.

#### Example 6

Effect of betaine application on the growth rate of pea

The experiment of Example 5 was repeated by utilizing betaine solutions of 0 (control), 0.05M, 0.1M and 0.2M. Water stress was induced in the manner described in Example 5, whereas salt stress was not examined in this experiment. In order to examine the recovery of plants, the stressed plants were divided on day 28 of the experiment into two groups one of which still remained under water stress, and the other one was irrigated and its recovery was followed. Samples were taken on days 21, 28, 35 and 42. Peas growing under optimum conditions (sufficient irrigation) were used as control. The best results were obtained with the betaine rate of 0.05M. The results concerning the relative growth rate of pea and the dry weight of the shoot are shown in Figures 1 and 2, respectively.

#### Example 7

Effect of betaine application on faba bean yield under stress conditions

The experiments described in Example 5 were repeated utilizing faba bean. Ten seeds of faba bean were sown per pot and they were later thinned to 3 plants per pot. The other parameters of the experiments corresponded to those described in Example 5.

The positive effect on faba bean was apparent especially for the number of pods, which increased under water stress from a control value of 3.13 to 3.50 with the 0.1M betaine solution, and to 3.63 with the 0.3M betaine solution. The results correspond to values 112 and 116 in percentages of the control (100). The leaf dry matter content increased from a control value of 2.04 g to 2.21 g with the 0.1M betaine solution, but decreased to 1.67 g with the 0.3M betaine solution.

#### Example 8

##### Effect of betaine application on soybean yield

The effect of betaine application on soybean yield under normal and dry conditions was investigated in field conditions on a farm where the soil is very light and sandy and can retain about 35 mm of rain for a few days, but where water stress will occur in a few days even after a heavy rain. There were overhead irrigation facilities on the farm to guarantee sufficient irrigation of the control areas. In addition to the growth of soybean, the experiment also determined its nitrogen fixation capacity.

The experiments were conducted with a 3-Factor Randomized Complete Block Design with watering level (main factor), cultivar (subplot) and betaine concentration (split) as factors. The cultivars were Biloxi and Cook, which have a different drought tolerance; cultivar Cook has a more drought tolerant symbiosis system than cultivar Biloxi. The betaine levels applied included 0 (control), 3 kg/ha, and 6 kg/ha. Betaine was applied by spraying. The season had an early drought period followed by very heavy rains and again a drought period. The betaine application was repeated after the rains before the second drought period with the same application levels. The results concerning the leaf dry weight are shown in Table 7.

**Table 7**  
**Effect of betaine application on the leaf dry weight of soybean**

betaine concentration (kg/ha)	leaf dry weight (g/plant)							
	Cook				Biloxi			
	normal	% of control	dry stress	% of control	normal	% of control	dry stress	% of control
0	13.5	100	11.1	100	15.1	100	10.5	100
3	14.1	104	12.6	113	16.3	108	11.5	110
6	17.7	131	10.2	92	15.5	103	10.6	101



The nitrogen fixation capacity of soybean was determined by measuring the nitrogenase activity with an acetylene reduction test wherein acetylene is reduced to ethylene. The experiment was conducted 10 weeks after sowing. In order to perform the measurement in the field, metal cylinders of 10 cm in diameter and 20 cm in depth were placed in the soil around a soybean plant. The plant was removed from the soil in the cylinder and the shoot was cut off. The roots were then quickly placed in an airtight container of 1000 ml. 150 ml of acetylene was then injected in the container, and a 6.5 ml gas sample was taken by a syringe 5, 10 and 15 minutes after incubation, and the samples were then subjected to gas chromatography. It has been established that acetylene reduction is linear for about 20 minutes from the acetylene introduction. The results obtained after 15 minutes are shown in Table 8.

Table 8

Effect of betaine application on the nitrogen fixation of soybean

	betaine concentration (kg/ha)	ethylene concentration ( $\mu\text{mol}/2$ plants)			
		Cv. Cook		Cv. Biloxi	
		dry stress	normal	dry stress	normal
	0 (control)	0.29	0.55	0.42	0.68
	3	0.60	0.68	0.53	0.79
	6	0.34	0.52	0.35	0.38

The application of betaine at the rate of 3 kg/ha thus clearly increases the nitrogen fixation of plants.

**Example 9****Effect of betaine application on soybean yield**

The acetylene reduction test described in Example 8 was repeated in greenhouses utilizing the betaine rate of 0.1M and cultivar Biloxi. The experiment was conducted by closing plant roots (2 per pot) in a glass container (1 l) and by sucking 150 ml of air out of the container, whereafter the air was replaced with a corresponding amount of acetylene gas in the manner described in Example 8. (Reference: Denison, R.F., Sinclair, T.R., Zobel, R.W., Johnson, M.N. & Drake, G.M. 1983. *A non-destructive field assay for soybean nitrogen fixation by acetylene reduction*. Plant & Soil 70; 173-182; Vessey, J.K. 1994. *Measurement of nitrogenase activity in legume root nodules; In defense of the acetylene reduction assay*. Plant & Soil 158; 151-162).

The nitrogen fixation of soybeans of 4 weeks was assayed in greenhouse tests 2 days after the betaine application. The results are shown in Table 9.

**Table 9**

Effect of betaine application on the nitrogen fixation of soybean

betaine concentration (M)	ethylene concentration (ppm)	
	normal	water stress
0 (control)	3.029	2.193
0.1	3.642	2.690

**Example 10****Effect of betaine application on soybean yield**

The effect of betaine application on the photosynthesis of soybean was examined in greenhouses utilizing simulated water stress conditions.

Six (inoculated) seeds of nodulating or 15 (inoculated) seeds of non-nodulating soybean cultivar were sown in 5 l plastic pots containing a mixture of peat, vermiculite and sand in the ratio of 1:2:1. After seedling emergence the plants were thinned to 3 per pot. Half of the pots used in the experiment contained the nodulating cultivar and half the non-nodulating cultivar. Water stress (pF3) was imposed on the plants 15 days after seedling emergence. The water-stressed plants were divided into three groups, one of which (control) was treated with distilled water, the second one was sprayed with betaine at the rate of 2 kg/ha and the third one with betaine at the rate of 6 kg/ha a day after stress imposition. The photosynthetic activity of the plants was determined with the Li-cor Li-1600-Steady State Porometer. The apparatus and its use are described in the following references: Campbell, G.S. 1975. *Steady-state diffusion porometers*. In: Measurement of stomatal aperture and diffusive resistance. Coll. Agric. Res. Center Bull. 809. p. 20. Washington State Univ. Pullman, Wash, and Bingham, G.E. & Coyne, P.I. 1977. *A portable, temperature-controlled steady-state porometer for field measurements of transpiration and photosynthesis*. *Photosynthetica* 11(1): 148-160. The results are shown in Table 10.

**Table 10**  
**Effect of betaine application on the photosynthetic activity of soybean**

betaine concentration (kg/ha)	photosynthetic activity ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )							
	nodulating cultivar				non-nodulating cultivar			
	normal	% of control	dry stress	% of control	normal	% of control	dry stress	% of control
0 (control)	33	100	10	100	33	100	9	100
2	39	120	18	180	33	100	26	289
6	36	110	15	150	37	112	26	289

Betaine considerably improved the photosynthetic activity of soybean both under normal and water stress conditions with both application rates.

5

**Example 11****Effect of betaine application on the growth of soybean**

The effect of betaine on the photosynthesis of soybean and on the water potential situation of soybean plant leaves was examined in greenhouses with the betaine concentrations of 0 (control), 0.05M, 0.10M and 0.15M. The soybean cultivar was Biloxi. At the time of spraying, the plants were about six weeks old and the measurements were performed five days after the spraying. The photosynthesis and the water potential of the plants, i.e. the stomatal resistance and stomatal conductance and the temperature difference of leaves were measured with the Li-cor Li 6200 Portable Photosynthesis System. The system is based on a method described by Ball et al. (*A Model predicting stomatal conductance and its contribution to the control of photosynthesis under different conditions*. Progress in Photosynthesis Research IV. Martinus Nijhoff, Dordrecht, The Netherlands, 1987, pp. 221-224, (Ed.) J. Biggins).

Water potential parameters indicate the state of opening or closing of the guard cells of the plant. High resistance and low conductance mean that the plant has a poor intake of carbon dioxide and is stressed. The numerical results are shown in Table 11, a graphical illustration is provided in Figure 3.

Table 11

Effect of betaine on the photosynthesis and leaf water potential of soybean

5	betaine concentration (M)	photo- synthesis ( $\mu\text{mol m}^{-2}\text{s}^{-1}$ )	stomatal conductance ( $\text{ms}^{-1}$ )	stomatal resistance ( $\text{s cm}^{-1}$ )	leaf tempe- rature diffe- rence
10	0 (con- trol)	2.70	0.02	18.16	-2.81
	0.05	4.01	0.05	11.21	-3.01
	0.10	14.01	0.26	6.30	-1.55
	0.15	12.83	0.12	4.46	-1.72

- 15 The results clearly show that adding betaine can reduce the stress of plants. The photosynthetic activity of the plant thereby increases, thus leading to a higher yield.

## Claims

5           1. Exogenous use of betaine to improve the  
yield of grain legumes.

          2. Exogenous use of betaine to reduce the  
antinutrient content of grain legumes.

          3. Use of betaine according to claim 2 to  
reduce the alkaloid content of lupin.

10           4. Use according to any one of claims 1 to 3,  
c h a r a c t e r i z e d    in that betaine is used  
under stress conditions.

          5. Use according to claim 4, c h a r a c -  
t e r i z e d   in that the stress conditions comprise  
15 high or low temperatures, drought or high salinity.

          6. Use according to any one of claims 1 to 5,  
c h a r a c t e r i z e d   in that betaine is used in  
an amount of about 0.1 to 20 kg/ha.

          7. Use according to claim 6, c h a r a c -  
t e r i z e d   in that betaine is used in an amount of  
20 about 1 to 6 kg/ha.

          8. A method for improving the yield of grain  
legumes, c h a r a c t e r i z e d   in that betaine is  
exogenously applied to growing grain legumes.

25           9. A method for reducing the antinutrient  
content of grain legumes, c h a r a c t e r i z e d  
in that betaine is exogenously applied to growing grain  
legumes.

          10. A method according to claim 9,  
30 c h a r a c t e r i z e d   in that the alkaloid content  
of lupins is reduced by exogenously applying betaine to  
growing lupins.

          11. A method according to any one of claims 8  
to 10, c h a r a c t e r i z e d   in that betaine is  
35 applied to grain legumes growing under stress conditions.

12. A method according to any one of claims 8 to 11, characterized in that the stress conditions comprise high or low temperatures, drought or high salinity.

5           13. A method according to any one of claims 8 to 12, characterized in that betaine is administered once or several times during the growing season.

10           14. A method according to any one of claims 8 to 13, characterized in that betaine is administered together with a pesticide or a fertilizer.

15           15. A method according to any one of claims 8 to 14, characterized in that betaine is administered in a single dose at an early stage of growth.

            16. A method according to any one of claims 8 to 15, characterized in that betaine is used in an amount of about 0.1 to 20 kg/ha, preferably about 1 to 6 kg/ha.

20           17. Grain legumes obtained with the method according to any one of claims 8 to 16, and the parts thereof, especially the seed yield.

            18. Grain legumes treated exogenously with betaine, and the products thereof.

25           19. An invention according to any one of claims 1 to 18, characterized in that the grain legume is lupin, pea, soybean, faba bean or green bean.



FIG. 1

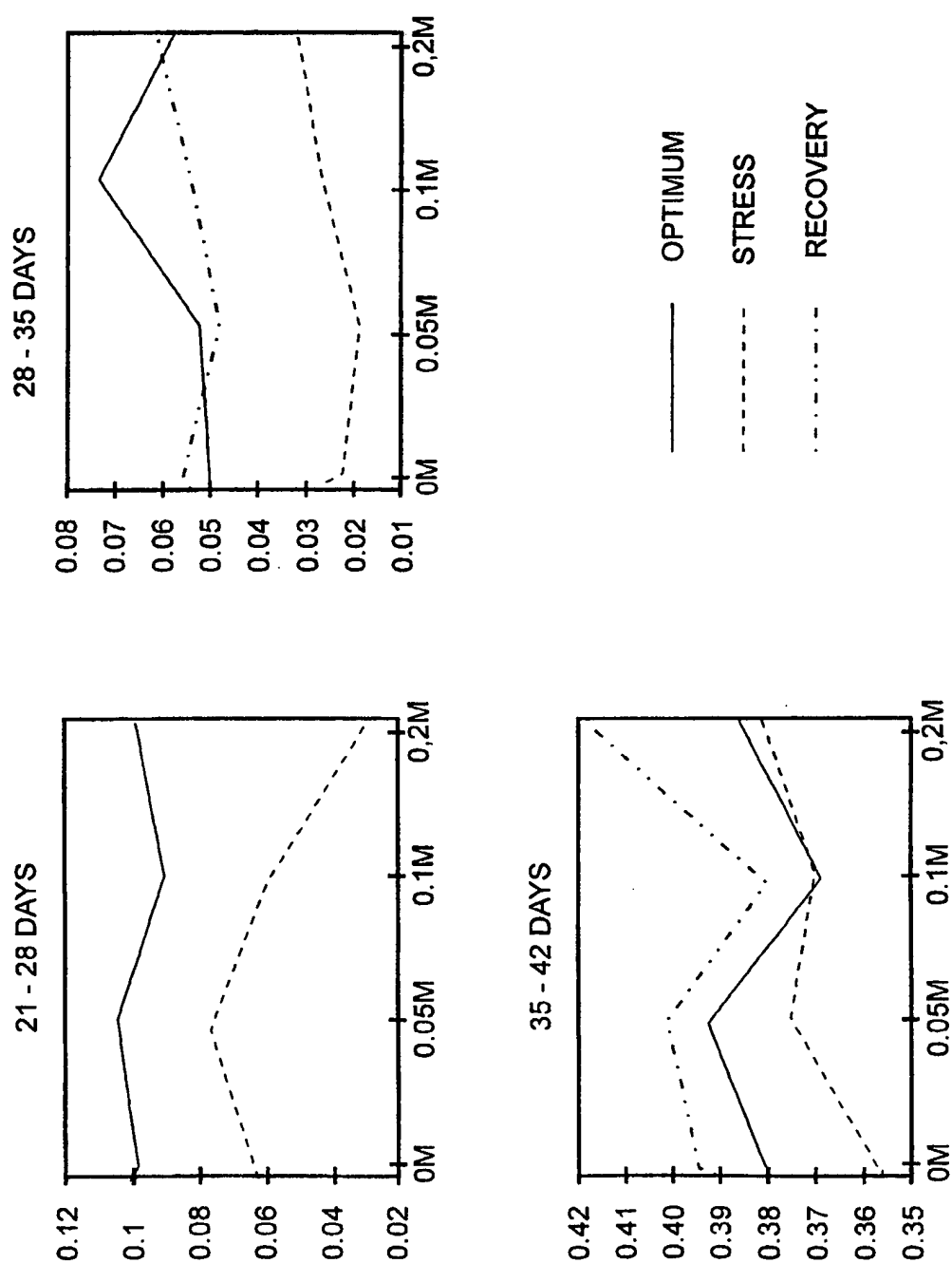


FIG. 2

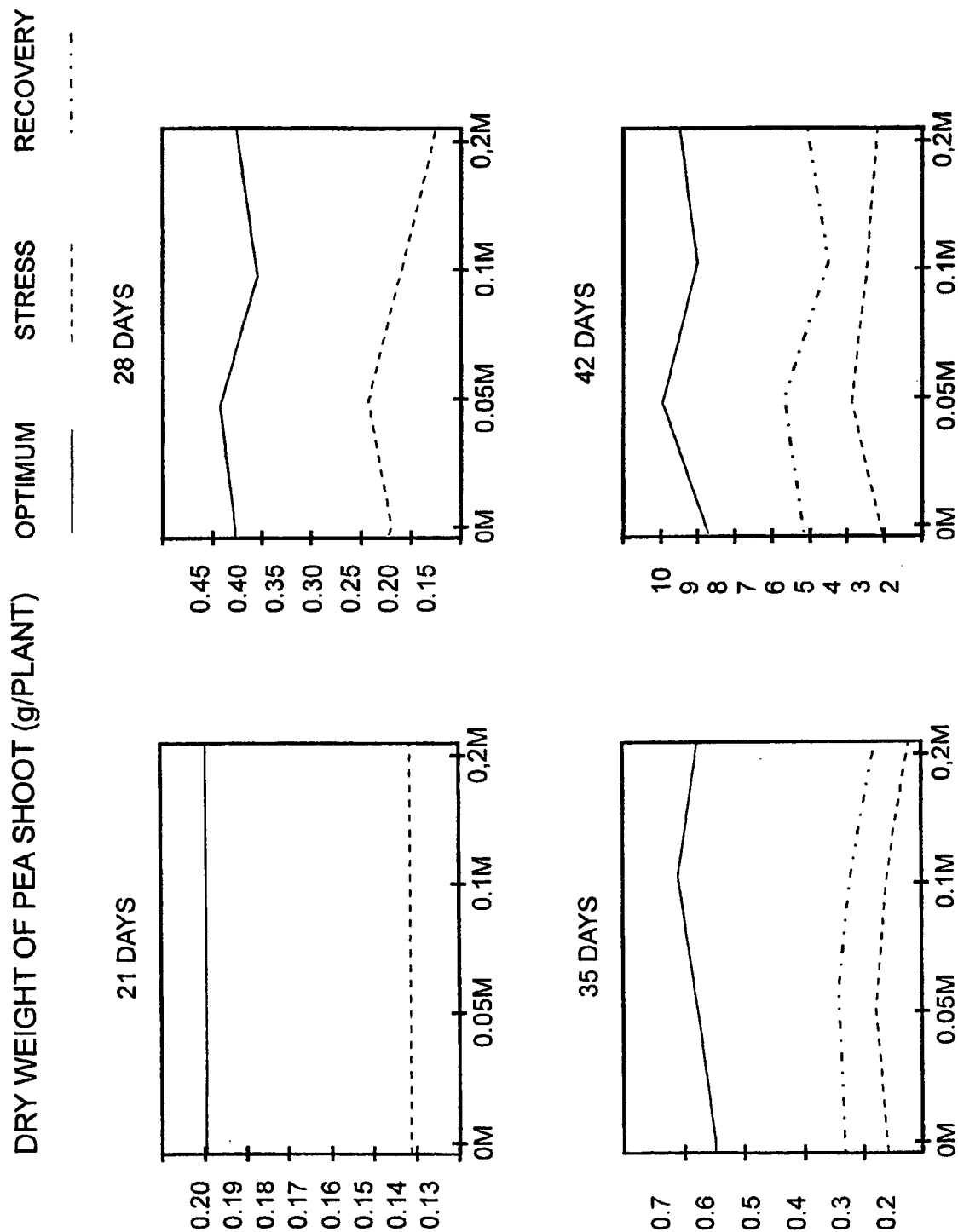
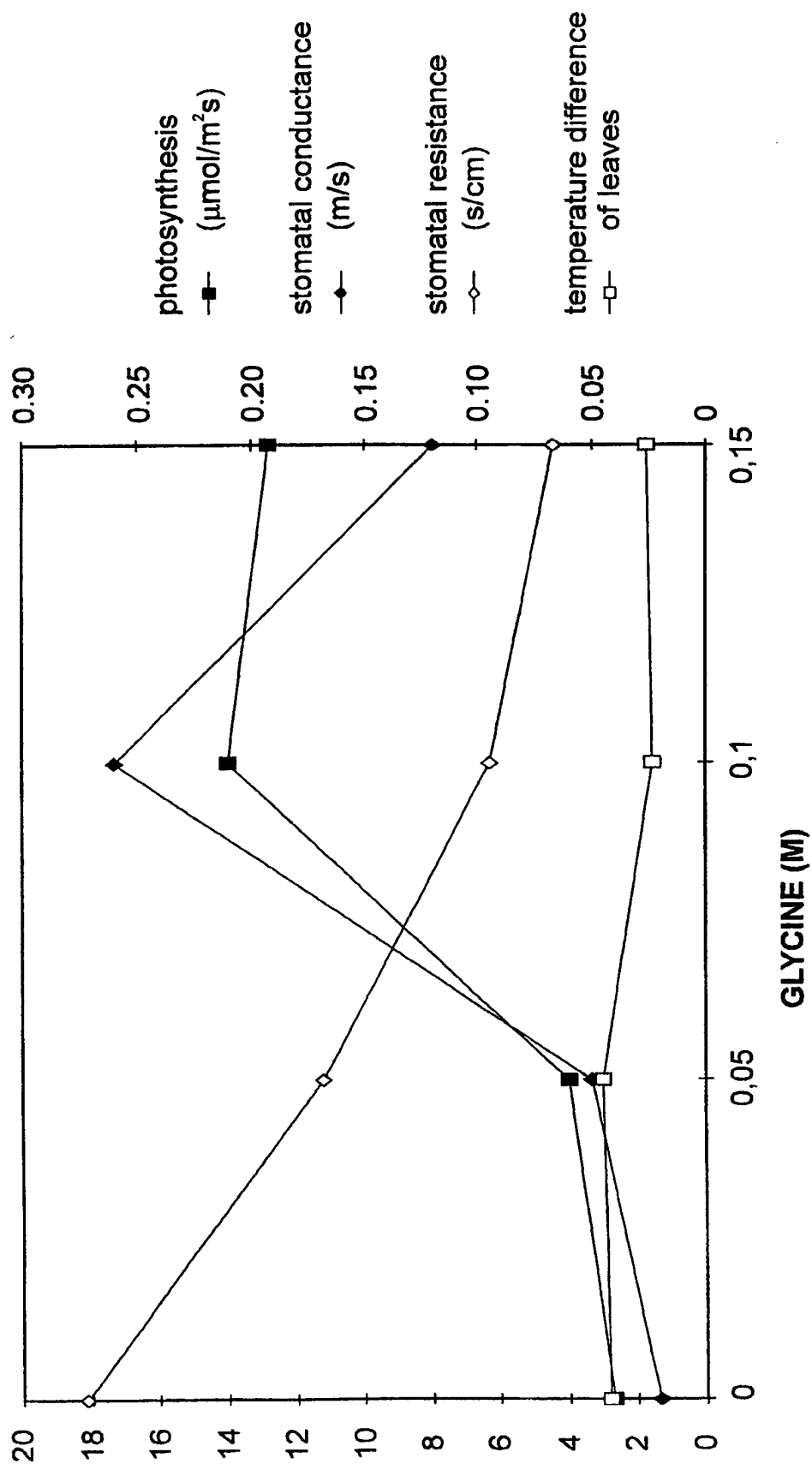


FIG. 3



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 95/00480

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A01N 37/44, A01N 33/12, A01G 7/00, C05C 11/00  
According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A01N, A01G, C05C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAPLUS, CAB ABSTRACTS, WPI, IFIPAT

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STN International, File WPIDS, WPIDS accession no. 89-312201, CHIKKARIN K: "Plant supported on basal bed - is cultivated with nourishing lig. contg. betaine (s)", & JP,A,01228416, 890912 (8943) --	1-19
X	EP 0181494 A1 (MITSUBISHI GAS CHEMICAL COMPANY, INC.), 21 May 1986 (21.05.86), page 6, compound No. 40, page 13, line 31 - page 14, line 22, page 17, line 13 - page 18, line 19, claims 1, 7, 11 --	1-19
X	JOURNAL OF EXPERIMENTAL BOTANY, Volume 38, No 188, March 1987, M. I. LONE et al, "Influence of Proline and Glycinebetaine on Salt Tolerance of Cultured Barley Embryos" page 479 - page 490 --	1-19

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

\* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
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"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

19 March 1996

Date of mailing of the international search report

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 95/00480

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PLANT SCIENCE LETTERS, Volume 25, 1982, C. ITAI et al, "Responses of water-stressed HORDEUM DISTICHUM L. and CUCUMIS SATIVUS to proline and betaine" page 329 - page 335 --	1-19
X	J. PLANT PHYSIOL., Volume 140, 1992, Y. ZHAO et al, "Protection of Membrane Integrity in Medicago sativa L. by Glycinebetaine against the Effects of Freezing" page 541 - page 543 --	1-19
X	STN International, File CABA, CABA accession no. 82:52208, Nash, D. et al: "Effect of proline, betaine and some other solutes on the heat stability of mitochondrial enzymes", & Australian Journal of Plant Physiology, (1982) Vol. 9, No. 1, pp. 47-57 -- -----	1-19

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/FI 95/00480

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0181494	21/05/86	AU-B,B- 556638	13/11/86
		AU-A- 4816485	17/04/86
		JP-A- 62045503	27/02/87
		JP-A- 62045504	27/02/87
		JP-A- 61091104	09/05/86
		JP-A- 61091105	09/05/86
		JP-A- 61091106	09/05/86
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